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(54) Heparin fragments having a selective anticoagulation activity and process for their preparation.

(57) Heparin fragments having selective anticoagulation activity having 14–18 sugar units, the disaccharide unit L-induronosyl-2-O-sulphate-N-sulpho-U-glucosamine-6-O-sulphate being the main component, and where unsulphated L-induronic acid is in a position situated 3–5 sugar units from the unreducing terminal. Pharmaceutical compositions containing such heparin fragments. Processes for the preparation of the heparin fragments.

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TITLE MODIFIED
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HEPARIN FRAGMENTS HAVING SELECTIVE ANTICOAGULATION ACTIVITY.

The present invention relates to heparin fragments which have
been shown to possess selective anticoagulation activity, a
process for the preparation thereof, and therapeutical
compositions containing such fragments.

Heparin is a sulphate-containing polysaccharide which on a
large scale is isolated from intestinal mucus from swine or
lung from cattle. It has for several decades been used
·clinically as an agent for the treatment and prevention of
thrombosis. In spite of the fact that the use of heparin in
thrombosis prophylaxis and therapy is still increasing, this
form of treatment is far from unproblematic. An important
problem is that the dosage must be balanced in such a manner
that a good thrombosis protection is obtained simultaneously
as bleeding complications are avoided. A difficulty in this
context is the great individual variation between different
patients; this is in turn probably dependent on the fact that
the heparin is bound to a varying degree to other components
in the blood plasma and thereby neutralised. Another problem
is that the preventive heparin treatment suffers from limited
success. A third problem with the present type of heparin is
its weak effect on arterial thrombosis. At said type of
thrombosis the thrombocyte aggregation is a more dominating
feature than at the venous thrombosis where heparin gives a
good effect. Standard heparin stimulates to a certain extent
thrombocyte aggregation and accordingly gives a negative
effect in said respect.

The mechanism of the anticoagulation activity of the heparin
is now essentially known. The blood coagulation is based on
a cascade like process where a number of proteolytic enzymes
are activating each other in a definite sequence; in the last
stage fibrinogen is converted under the action of the pro-
teolytic enzyme thrombin to insoluble fibrin, the foundamental
structure in a blood coagel. Heparin forms a complex with
a plasma protein, and this complex inhibits most of the
enzymes in the coagulation cascade.

It has been recently shown that heparin fractions of different
molecular weights influence the coagulation process in
different ways /L.-O. Andersson et al, Tromb. Res. 9, 575
(1976)7. This initiated a study of the possibilities to
develop heparin fractions having a more selective action.
Treatment of standard heparin with nitrous acid in dimethoxy-
ethane (glyme) at a low temperature and for a certain definite
period of time has resulted in a special fragment of heparin
possessing a considerably higher selective action than
standard heparin. This heparin derivative has a very small
effect on the inhibition of thrombin, while the inhibition of
activated coagulation factor X is highly accelerated.
Coagulation factor X takes a central position in the middle
of the coagulation cascade and the inhibition thereof is by
many considered especially important to obtain an effective
thrombosis-preventing effect /S. Wessler, Thromb. Diath.
Haemorrh. 33, 81 (1974)7.

It has further quite unexpectedly been shown that said type
of fragments are not neutralised by the blood components to
the same extent as standard heparin. This results in, inter
alia, a more efficient utilisation of the anticoagulation
activity of this type of fragments compared to the present
clinically used heparin preparations. Further, also the
dosage is easier to perform, as the individual variation of
heparin-neutralising effect is less important to take into
consideration. A further surprising property of the fragment
is that its thrombocyte aggregation-inducing activity is much

lower than that which is usually shown by heparin. Therefore,
it is probable that this type of fragment is a better anti-
coagulation agent than standard heparin for preventive
treatment and treatment of arterial thromboses. It may also
be assumed that the reduced influence on the thrombocytes may
lead to a lowered risk of bleeding complications.

It should also be noted that the capacity of the heparin to
release the enzyme lipoprotein lipase is strongly dependent
on the molecular weight. Therefore, it may be assumed that
the low molecular weight heparin fragment has a further
valuable property in that it to a lesser extent than standard
heparin increases the contents of free fatty acids in the
blood.

This special type of heparin fragments may be prepared in
several different ways. One of the methods a) comprises treat-
ment of standard heparin with nitrous acid in dimethoxyethane
as mentioned above. Said method gives this type of fragments
together with a series of inactive fragments. The active
fragments may then be freed from inactive elements, such as
by affinity chromatography on matrix-bound antithrombin III
/Höök et al., FEBS Lett. 66, 90 (1976); Hopwood et al., FEBS
Lett. 63, 51 (1976); L.-O. Andersson et al., Thromb. Res. 9,
575 (1976)/. Other ways of preparing fragments are: b) via
periodate oxidation at low pH and low temperature; c) via
partial depolymerisation with heparinase; d) via partial
depolymerisation of heparin by esterification of carboxyl
groups and subsequent alkaline β-elimination; e) via partial
depolymerisation of heparin by partial N-desulphatation and
subsequent deamination with nitrous acid at a pH value of
3.9. Methods a) and b) are described in the examples.

The active fragments are characterised in that they contain
from 14 to 18 sugar units. Structural analysis shows the
same main structural components as in standard heparin, i.e.
L-iduronosyl-2-O-sulphate-(1α-4)-N-sulpho-D-glucosamine-6-O-
-sulphate as the dominating saccharide unit. However, the

amount of unsulphated iduronic acid is considerably higher
than in the starting material. Periodate oxidation has shown
that this component takes a definite position in the molecule
situated from 3 to 5 sugar units counted from the unreducing
terminal. The active fragments have the structure
$(U-G)_n-I-G-(U-G)_m$ where n is 1 or 2 and m is 5 or 6, I is
unsulphated L-iduronic acid, U is L-iduronic acid-2-O-sulphate
and G is N-sulpho-D-glucosamine-6-O-sulphate. A few U units
may lack O-sulphate or be replaced by D-glucuronic acid and,
similarly, a few G units may lack O-sulphate or be replaced
by N-acetyl-D-glucosamine units. Reducing or unreducing
terminal units may vary with the type of method of preparation
used; thus, e.g., deaminitive splitting of heparin leads to
the formation of 2,5-anhydro-D-mannose in reducing terminal
position. The active fragments may be characterised by means
of physico-chemical methods, such as determination of mobility
in an electric field and UV, IR and NMR spectra. However,
the numerical values obtained do not give complete information,
as also coagulation-inactive fragments substantially show
similar characteristics. This depends on the fact that the
biologic activity is derived from a specific sequence of the
sugar residues where the position of the unsulphated uronic
acid is especially important. Thus, only a gross composition
and size does not warrant that the component is active.

The invention is further illustrated by the following
examples.

Example 1.

Preparation of heparin fragments by depolymerisation of
standard heparin with nitrous acid.

Heparin (0.5 g) isolated from swine intestines and dissolved
in 150 ml of water is chilled to +4°C. and brought to pass
through a 3 x 7 cm column of Dowex ® 50 W-28 (H⁺ form),
200-400 mesh. The column is then washed with 100 ml of water
and the washing liquid is combined with the sample. To the
sample there are added 250 ml of dimethoxyethane (glyme)
chilled to -20°C. and 10 ml of isoamyl nitrite and the

mixture having a temperature of about $+10^{\circ}C$. is allowed to
stand for two minutes. The reaction is then discontinued by
the addition of 10 ml of 10% Na^{+}-acetate. After addition of
5.2 liters of ethanol precipitated carbohydrate (heparin
derivatives) is recovered by centrifugation. The product is
dissolved in 500 ml of 0.05M NaCl - 0.05M Tris-HCl, pH 7.4.
This solution is fractionated, divided into 100 ml portions,
by affinity chromatography on a column containing 75 ml of
antithrombin-Sepharose ® (Pharmacia Fine Chemicals,
Uppsala, Sweden) (about 5 mg of protein per ml gel). The
column is eluated by a salt gradient (500 ml of 0.05M NaCl -
0.05M Tris-HCl in the mixing vessel; 500 ml of 3M NaCl -
0.05M Tris-HCl in the reservoir), the major part of the applied
material either passing unretardedly through the column or being
eluated at a low ion strength (less than 0.4M NaCl); this
material has no biologic activity. The active components
(purified heparin derivatives) are eluated in a
wide top 0.5M NaCl and 3M NaCl corresponding to about 4% of
the starting material. These fractions are pooled and
desalted by gel chromatography.

Heparin derivatives prepared and purified in said manner have
a molecular size corresponding to that of a tetradeca-octa-
decasaccharide (molecular weight 3600-4800). Structural
analysis show the same structural components as in the
starting material, L-iduronosyl-2-O-sulphate-(1α-4)-N-sulpho-
-D-glucosamine-6-O-sulphate being the dominating disaccharide
unit. However, the amount of unsulphated iduronic acid has
increased from about 6% in the starting material to about
16% . As to other aspects the structure agrees with the
above description.

Example 2.
Partial depolymerisation of heparin or heparin by-product by
periodate oxidation at a pH value of 3 and $4^{\circ}C$. and subsequent
alkali-treatment and reduction.
Under these conditions the polysaccharide chain is split at
D-glucuronic acid units resulting in only moderate loss of

anticoagulation activity (Fransson and Lewis, FEBS Letters, 1979, in press). Standard heparin (0.5 g) is dissolved in 250 ml of a solution (4°C.) containing 0.02M $NaIO_4$, 0.2M $NaClO_4$ and 0.05M Na^+-citrate buffer, pH 3.0.. After three hours incubation in dark at +4°C. the oxidation is discontinued by the addition of a molar excess of D-mannitol and, then, the solution is dialysed and freeze-dried. Splitting of the polysaccharide chains at oxidised D-glucuronic acid units is effected by treatment of the product with alkali (5 mg/ml of aqueous solution adjusted to pH 12 with 1M NaOH) at room temperature. After ten minutes the solution is neutralised with 1M acetic acid and, then, the material is desalted by gel chromatography on dextran material (Sephadex ® G-25, Pharmacia Fine Chemicals, Uppsala, Sweden). The product obtained can be reduced with sodium borohydride.

Heparin treated in said manner is considerably depolymerised compared to the starting material; gel chromatography shows that the resulting fragments have a size corresponding to 10-25 sugar units and, therefore, they are somewhat larger than the fragments isolated after treatment with nitrous acid according to Example 1. Totally about 20% of the uronic acids in the polysaccharide are destroyed under the periodate oxidation (pH 3, 4°C., 3-6 hours). Purification by affinity chromatography of the oxidation products on antithrombin- -agarose-Sepharose ® gave a yield of about 30% of high-affinity material after three hours and a yield of 15% after six hours oxidation. The products thus obtained had an antifactor X_a-potentiating effect determined according to Example B in plasma of more than 1000 units/mg compared to 3rd International Heparin Standard.

Studies on anticoagulation activity.
The heparin fragment prepared according to Example 1 was studied in view of its capacity to: A) accelerate the inhibition of the coagulation enzyme thrombin; B) accelerate the inhibition of activated coagulation factor X; C) prolong the coagulation time in the blood plasma coagulation test

APTT (Activated Partial Thromboplastine Time); D) be
neutralised by blood plasma components; and E) influence the
aggregation of thrombocytes.

Example A.
Inhibition of thrombin.

The capacity of the heparin fragment to potentiate the inhi-
bition of thrombin with antithrombin III was analysed
according to a modification of a method by Teien et al.
(Thrombosis Research 11, p. 107-117, 1977). The heparin
fragment was found to have a specific activity of less than
20 E/mg compared to 120-170 E/mg for standard heparin.

Example B.
Inhibition of activated factor X.

The capacity of the heparin fragment to potentiate the
inhibition of activated factor X in plasma and in pure anti-
thrombin III was studied according to a modified version of a
method by Teien et al. (Thrombosis Research 8, 413, 1976).
The heparin fraction was shown to have a specific activity of
500 E/mg in a pure antithrombin III system and 2100 E/mg in
a plasma system compared to 120-170 E/mg for standard heparin.

Example C.
Prolongation of the coagulation time.

The capacity to prolong the coagulation time of blood plasma
was studied according to the APTT (Activated Partial
Thromboplastine Time) method /Andersson et al., Thromb. Res.
9, 575 (1976)/. The heparin fragment showed a specific
activity of less than 20 E/mg compared to 3rd International
Heparin Standard. Standard heparin shows a specific activity
in the range 120-170 E/mg.

Example D.
Neutralisation of heparin fragments in blood plasma.

The heparin-neutralising effect of plasma components was
studied by measuring the effect of heparin and the heparin
fragment in plasma and in a pure antithrombin system. This

was performed by measuring the amount of activated factor X inhibited in the two systems in the presence of a certain amount of heparin or heparin fragment. The activity of the heparin fragment showed a 15% neutralisation by plasma components, while the corresponding value of standard heparin was shown to be 75%.

Example E.

Thrombocyte influence.

The capacity of the heparin fragment to aggregate thrombocytes at critical ADP (Adenosine DiPhosphate) concentrations was substantially studied according to Beck, E.A. (Thromb Haem Stuttg. 1977, 38, 578). It was shown that the thrombocyte-aggregating capacity of the heparin fragment was ten times lower than that of standard heparin, calculated on the weight.

The heparin fragment according to the invention is incorporated into pharmaceutical preparations for clinical use, preferably in aqueous solution for injection or in ointment preparations for administration via the skin and mucous membranes.

CLAIMS.

1.   Heparin fragments, characterised by 14-18 sugar units, the disaccharide unit L-iduronosyl-2-O-sulphate-N-sulpho-D-glucosamine-6-O-sulphate being the main component, and where unsulphated L-iduronic acid is in a position situated 3-5 sugar units from the unreducing terminal and is followed by N-sulpho-D-glucosamine sulphate or N-acetyl-glucosamine in sulphated or unsulphated form.

2.   Heparin fragments, characterised by the structure $(U-G)_n-I-G-(U-G)_m$ where n is 1 or 2 and m is 5 or 6, I is unsulphated L-iduronic acid, U is L-iduronic acid-2-O-sulphate, and G is N-sulpho-D-glucosamine-6-O-sulphate.

3.   Pharmaceutical compositions, characterised in that they comprise heparin fragments having selective anti-coagulation activity and containing 14-18 sugar units, the disaccharide unit L-iduronosyl-2-O-sulphate-N-sulpho-D-glucosamine-6-O-sulphate being the main component, and where unsulphated L-iduronic acid is in a position situated 3-5 sugar units from the unreducing terminal and is followed by N-sulpho-D-glucosamine sulphate or N-acetyl-glucosamine in sulphated or unsulphated form.

4.   Pharmaceutical compositions, characterised in that they contain heparin fragments of the structure $(U-G)_n-I-G-(U-G)_m$ where n is 1 or 2 and m is 5 or 6, I is unsulphated L-iduronic acid, U is L-iduronic acid-2-O-sulphate and G is N-sulpho-D-glucosamine-6-O-sulphate.

5.   A process for the preparation of heparin fragments according to claim 1, characterised by
(a)   treating heparin with nitrous acid in dimethoxyethane and purifying the material obtained, or
(b)   periodate-oxidising heparin at low pH and temperature, respectively, or
(c)   partially depolymerising heparin with heparinase,

or

(d)   partially depolymerising heparin by esterification
of carboxyl groups and then subjecting the material
obtained to alkaline β-elimination, or

(e)   partially depolymerising heparin from partial
N-desulphatation and then deaminating the material
obtained with nitrous acid.